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1) Curr Opin Biotechnol. 1990 Oct;1(1):36-47.

Mammalian cell expression.

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2) Biotechnology (N Y). 1990 Jan;8(1):54-8.

Spin filter perfusion system for high density cell culture: production of recombinant urinary type plasminogen activator in CHO cells.

Avgerinos GC, Drapeau D, Socolow JS, Mao JI, Hsiao K, Broeze RJ.

3) Biotechnology (N Y). 1995 Apr;13(4):389-92.

Production of recombinant proteins in Chinese hamster ovary cells using a protein-free cell culture medium.

Zang M, Trautmann H, Gandor C, Messi F, Asselbergs F, Leist C, Fiechter A, Reiser J.

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Kaufman RJ, Wasley LC, Spiliotes AJ, Gossels SD, Latt SA, Larsen GR, Kay RM.

Thank you,

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SPIN FILTER PERFUSION SYSTEM FOR HIGH DENSITY CELL CULTURE: PRODUCTION OF RECOMBINANT URINARY TYPE PLASMINOGEN ACTIVATOR IN CHO CELLS

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We have used a 20 liter stirred tank fermentor, equipped with a 127 mesh ethylene-tetraflouroethylene rotating screen for cell recycle, for the continuous production of recombinant single chain urokinase-type plasminogen activator (rscu-PA) from Chinese hamster ovary (CHO) cells. Viable cell densities between 60 and 74 million per ml were maintained at medium perfusion rates of 3.0 to 4.0 fermentor volumes per day. Cells were retained by the 120 micron nominal opening filter through the formation of "clumped" cell aggregates of 200 to 600 μm in size, which did not foul the filter. In 31 days of culture, a total of 51 grams of rscu-PA were produced in 1,000 liters of medium. The rscu-PA produced over the course of this continuous culture was purified and characterized both *in vitro* and *in vivo* and shown to be comparable to natural scu-PA produced from the transformed human kidney cell line, TCL-598.

Chinese hamster ovary (CHO) cells have been used for the production of a number of heterologous proteins of human therapeutic value such as beta and gamma interferon, factor VIII, tissue plasminogen activator and others[1–5]. These properly processed, biologically active proteins with "mammalian-like" glycosylation patterns are readily expressed and secreted into the medium by CHO cells[4].

The CHO host/vector system currently most frequently used relies on co-amplification of the genes for dihydrofolate reductase (DHFR) and the product of interest in $DHFR^-$ host cell lines[6]. A strong constitutive viral promoter drives the desired gene expression and results in secretion of product at a rate independent of the growth rate of the cells. Rates as high as 45 μg per 10^6 cells/day have been reported[3,5,7].

CHO cell lines used for such heterologous protein production have been grown in either suspension or in a variety of attached culture systems. However, in either type of cell cultivation system, the cell density, resulting product titer and reactor volumetric productivity achieved are ultimately limited by the supply of nutrients and the accumulation of inhibitory metabolites at the cell surface.

Various perfusion systems, in which cells are retained in a bioreactor while conditioned medium is constantly replaced with fresh medium, have been used to increase cell density, and hence, reactor productivity. Examples are hollow fiber bioreactors[8], stirred tank bioreactors with an external membrane cell separator[9,10], porous weighted spheres in a fluidized bed[7], porous ceramic matrices[11], and packed bed bioreactors[12].

Perhaps one of the simpler perfusion systems which can be used to grow mammalian cells is the stirred tank fermentor fitted with a rotating "spin filter". This concept was originally demonstrated by Himmelfarb and co-workers who showed a substantial increase in cell density in small scale continuously perfused versus batch cultures[13]. The constant rotation of the 3 micron filter used by these investigators greatly reduced the rate at which it fouled. However, adoption of this technology for manufacturing has been hampered in part by the need to identify the appropriate conditions for predictably minimizing filter fouling during scaled up operations with each cell line and medium condition used. Tolbert et al. scaled up spin filter suspension culture operation to the 40 liter scale with a 1 micron porous ceramic spin filter to achieve cell densities of $1\text{-}5\times10^7$/ml in fermentation runs with carcinosarcoma cells lasting two weeks[14]. These investigators subsequently further improved the process by moving the spin filter to a small external vessel to facilitate filter maintenance without terminating the fermentation[15]. Reuveny et al. grew 10–20 micron diameter hybridoma cells to densities of 2×10^7/ml with a slightly larger 5 micron stainless steel mesh screen[16]. However, this screen was shown to foul after approximately 7 days of operation. Fermentation was continued by periodically transferring the culture to a fresh fermentor. Still larger, 10 micron stainless steel screens were used by van Wezel et al. for fermentations of hybridoma cells[17]. Cell densities of $1\text{-}2\times10^7$/ml were achieved in 20 to 30 day fermentations. More recently, however, Scheirer and Varecka used stainless steel filters with 53 micron mesh openings, approximately twice the diameter of a single cell for continuously perfused fermentations[18,19]. This improvement prevented fouling of the filter and still resulted in 93–99% retention of cells. In our laboratory we have grown the transformed kidney cell line (TCL-598) for the continuous production of scu-PA in 10 to 200 liter fermentors equipped with 53 micron stainless steel mesh screens for periods of 50 to 100 days[20].

Coarser mesh screens with 50–150 micron openings have also been used to make rotating filters for perfusing cultures of anchorage dependent cell lines growing on various types of microcarriers in stirred tank culture[22]. However, increasing the microcarrier bead density and scaling up operating volumes to achieve higher cell densi-

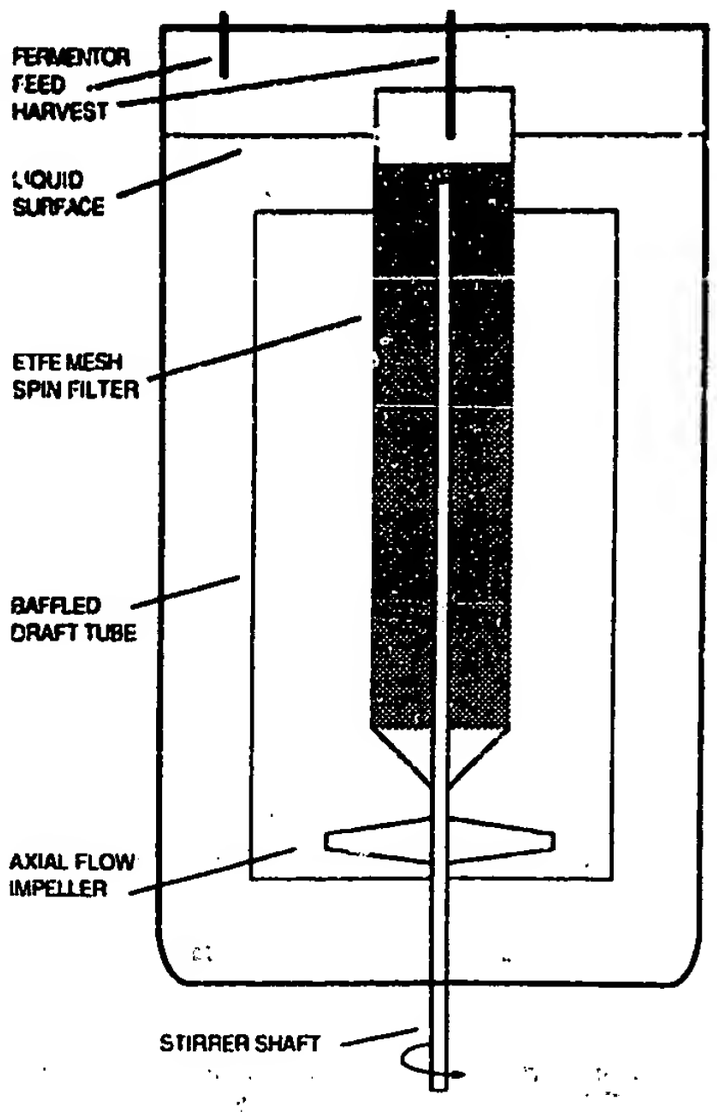

FIGURE 1 Schematic diagram of a 20 liter fermentor (10 liter working volume) equipped for continuous perfusion culture.

ties and reactor productivities have proven challenging[23,24].

In this report we describe the successful adaptation of spin filter technology to CHO cells for production of recombinant single chain urinary type plasminogen activator (scu-PA) at high cellular and volumetric productivities. To prevent fouling of the spin filter we have used a large-pore (120 μm) polymeric ethylene-tetrafluoroethylene (ETFE) screen. In order to retain 99% of the cells and achieve high cell densities, we took advantage of the propensity of CHO cells to grow with a clumped or aggregated cell morphology. The cells were grown in such aggregates which ranged from 200 to 600 microns in size. These clumped cells had similar specific productivities to non-clumped cell cultures, were self-propagating and were rapidly formed from single cells by using various microcarriers as initiating supports.

RESULTS AND DISCUSSION

Perfusion culture. A 20 liter Chemap (S. Plainfield, N.J.) fermentor with a 10 liter working volume was equipped for perfusion as shown in Figure 1. The bottom driven fermentor was fitted with a high efficiency axial-flow impeller and a draft tube. Retention of cells was achieved by using a 600 cm^2 127 mesh ETFE screen mounted on a cylindrical support on the impeller shaft. Fresh medium was continuously fed into the top of the vessel as conditioned medium was removed from the liquid surface inside the spin filter via a tube inserted through the top of the fermentor.

The fermentor was inoculated with cells of CHO clone CGM-113 attached to Cytodex 2 microcarrier beads at a final concentration of 5 g dry weight/liter. The initial cell density was 1.2×10^6 cells/ml (Fig. 2A). Perfusion was begun on the second day of fermentation at a rate of 0.6 liters per day using CRI-G3 medium, a low cost DMEM:F12 basal medium containing 0.5% fetal bovine serum, 5 μM methotrexate (MTX) and additional growth supplements. The perfusion rate was periodically adjusted to obtain a specific medium delivery rate of approximately 0.10 ml per 10^6 cells per day. A perfusion rate of 40 liters per day was reached on day 11 and was maintained thereafter. The viable cell density reached 1.0×10^7/ml within seven days of inoculation and 7.4×10^7/ml after sixteen days. As shown in Figure 2B, the total concentration of recombinant(r) single chain (sc) and two chain (tc) urokinase type-plasminogen activator (u-PA) in the conditioned medium peaked at 100 mg/l on day 20. Aprotinin, which has been shown to effectively prevent cleavage of plasminogen activators such as t-PA to their two chain forms[25] was included in the media at a concentration of 10 KIU/ml to maintain 80–90% of the rscu-PA in the single chain zymogen form (Fig. 2B). Fermentations conducted with medium without aprotinin typically resulted in zymogen levels of 40–50% due to proteolytic cleavage of rscu-PA after Lys-158 (data not shown). This fermentation was continued for a total of 31 days during which time a total of 1,000 liters of medium was perfused. A total production of 64 gms of ru-PA, of which 51 grams remained in the form of rscu-PA, was measured in the harvest. Thus, the average rscu-PA titer over the course of the experiment was 51 mg/liter.

Cell retention by the spin filter. The screen used to construct the spin filter had a nominal mesh opening of 120 microns, which was significantly larger than the 10–20 micron diameter of a single CHO cell. The CHO cells were initially retained by the spin filter because they were attached to the 115–195 micron diameter Cytodex 2 microcarrier beads. As the cells grew over the surface of the beads, aggregation of cells between beads began to occur. As shown in Figure 3, the cells began to aggregate after nine days of fermentation by which time the viable cell density had reached 2.9×10^7/ml. As cell growth continued, these cell aggregates enlarged and cells migrated off of the surface of the microcarrier beads. After 18 days only fully aggregated "clumped" cells and bare microcarrier beads remained in the reactor. These cell clumps, whose diameters ranged from 200 to 600 μm, were effectively retained by the 120 μm average pore size filter. For example, on day 11 the viable cell density was 4.1×10^7/ml as determined by trypan blue dye exclusion. The concentration of single cells or those in groups of 2 to 3 was estimated by hemocytometer to be only 3.8×10^5/ml. Apparently these unclumped cells readily passed through the spin filter as shown by the presence of cells in the fermentor harvest at a density of 2.7×10^5/ml. This specific rate at which cells were lost through the screen was 0.026 $days^{-1}$ and was small in comparison to the net specific growth rate of the culture (0.26 $days^{-1}$).

Fermentations were attempted with stainless steel mesh spin filters ranging from 44 to 105 microns in nominal screen opening size. The spin filters on four such attempts became plugged with cells after only 11 to 21 days of operation. Microscopic examination of these filters revealed that cells had attached to and subsequently grown on both sides of the stainless steel mesh. Five fermentations were subsequently conducted with ETFE filters using several different CHO clones. Fermentations up to 54 days in duration were achieved without filter fouling (data not shown). The hydrophobic nature of the ETFE screen apparently minimized the attachment of cells to the screen and thus greatly extended the life of the spin filter[26].

Cell growth. Using CRI-G3 medium, an average cell doubling time of 20–30 hours was observed for CHO cell line CGM-113 when grown in T-flasks at cell densities between 0.2–2.4×10^6/ml. In the fermentor, an initial 35 hour doubling time was observed at cell densities from 1.8 to 12×10^6/ml. The doubling time increased to 69 hours when the density reached 12×10^6/ml. This change coin-

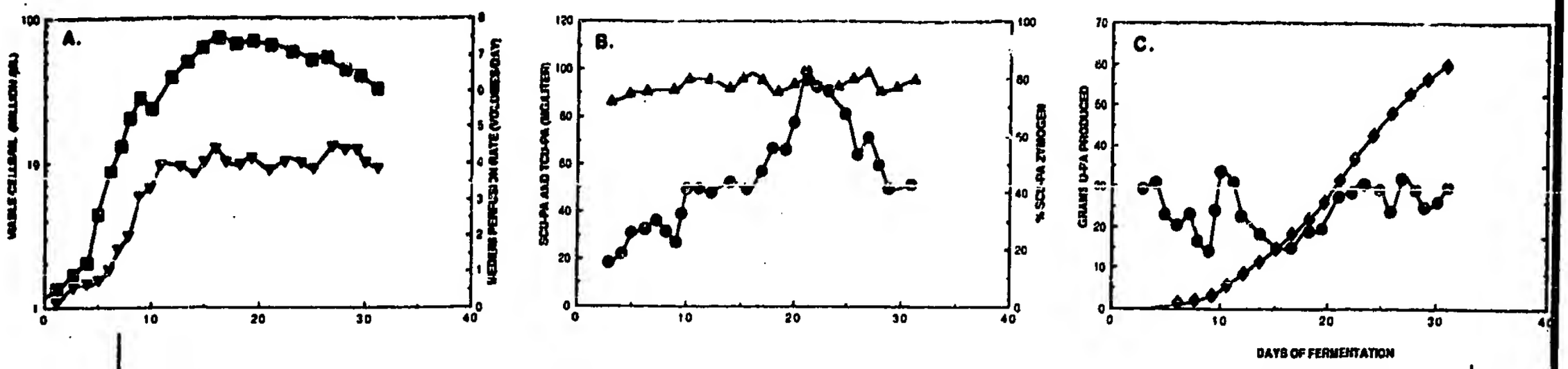

FIGURE 2 Results of a 20 liter continuous perfusion fermentation with clone CGM-113. Panel A: Viable cell count ($\times 10^6$ cells/ml) (■) and medium perfusion rate (▼) (expressed in volumes of medium per fermentor volume per day). Panel B: Total rscu-PA and rtcu-PA levels (mg/l) in the fermentor (●) and % of total in rscu-PA zymogen form (▲). Panel C: Total gms scu-PA produced (◆) and specific activity of cellular production (IU of total u-PA/10^6 cells/day) (●).

cided with the change in cell morphology from microcarrier growth to "clumped" cell growth which was independent of the microcarrier beads (Fig. 2A).

In order to scale up from the 20 liter level, it was useful to show that the microcarrier beads were not essential to the fermentation process. A fermentation which had been initiated with 5 g/l of microcarriers achieved a density of 2.0×10^7 aggregated cells/ml (clone CGM-34) after 35 days of operation. A second 20 liter fermentor was inoculated with 200 mls of this culture at a final cell density of 4×10^5 aggregated cells per ml. A 50 fold reduction in microcarrier concentration to 0.1 g/l was achieved by this dilution. The second fermentation was conducted for an additional 28 days during which time these clumped cells continued to grow achieving cell densities and growth rates similar to those observed in the first fermentation (data not shown). Thus, once initiated, the CHO cell clumps could be continuously propagated in a stirred tank fermentor independent of beads.

Cell viability in the fermentor. In order to count the total number of viable cells in the fermentor, the cell aggregates were trypsinized and stained with trypan blue. The viability of cells examined after trypsinization was found to be greater than 90% at all times in the fermentation.

In order to estimate cell death and lysis occurring within the large clumps in the fermentor, the levels of intra- and extracellular lactate dehydrogenase (LDH) and phosphohexose isomerase (PHI) were measured in sonicated cell pellets and centrifuged medium supernatants at various times during the fermentation. Average levels of intracellular LDH and PHI were determined to be 551 and 169 IU/10^9 viable cells respectively. Extracellular levels of these enzymes ranged from 150–700 IU/liter for LDH and 100–250 IU/liter for PHI depending on cell density and perfusion rate. These data were used to estimate specific cell death rates over the course of the fermentation. Average values of 0.058 ± 0.029 day^{-1} using extracellular LDH levels and 0.091 ± 0.026 day^{-1} using extracellular PHI levels were obtained. The data suggests that on average, a basal cell turnover rate with a doubling time equivalent to 180–280 hours was occurring in cell clumps in the fermentor.

Volumetric and specific productivity. Typical batch T-flask cultures of 3–5 day duration with clone CGM-113 achieved an average of $3–4\times10^6$ viable cells/ml with rscu-PA titers of 20–30 mg/liter in CRI-G3 medium. At high cell density in continu usly perfused culture, 51 mg/liter rscu-PA at 3–4 react r volumes p r day, a substantial improvement (>10 fold) in volumetric productivity, was achieved. Even though the cells in the continuously perfused ferment r grew in clumps of 200 to 600 microns in size, their specific ru-PA productivity appeared to be similar to that obtained with cells grown in monolayer in batch culture in T-flasks or roller bottles. As shown in Figure 2C, the average specific productivity of the cells in continuously perfused culture varied between 500–1,000 IU scu-PA (3.5–7.0 μg)/10^6 cells/day. These values were essentially the same as determined in batch T-flask cultures for the cell population used to seed the fermentor, ie. 750–1000 IU (5.2–7.0 μg) of scu-PA/10^6 cells per day.

Cells were removed from two additional fermentations for further characterization after 28 and 45 days of cultivation. After propagation of these cells as monolayer cultures in replicate T-flasks, the specific ru-PA productivity of the populations was determined. The specific productivity for the cells growing in T-flasks was equivalent to that found for the cells growing in the fermentor at the time they were harvested.

Purification of scu-PA. Recovery of rscu-PA from these fermentations was achieved by continuously harvesting the conditioned medium into chilled (4°C) 200 liter tanks. Batches of conditioned media representing seven days of harvest were purified using a four step purification procedure. The overall recovery observed for this purification procedure was approximately 40%. Analysis by SDS-PAGE of the material recovered after each purification step is shown Figure 4.

The purified rscu-PA had an apparent molecular

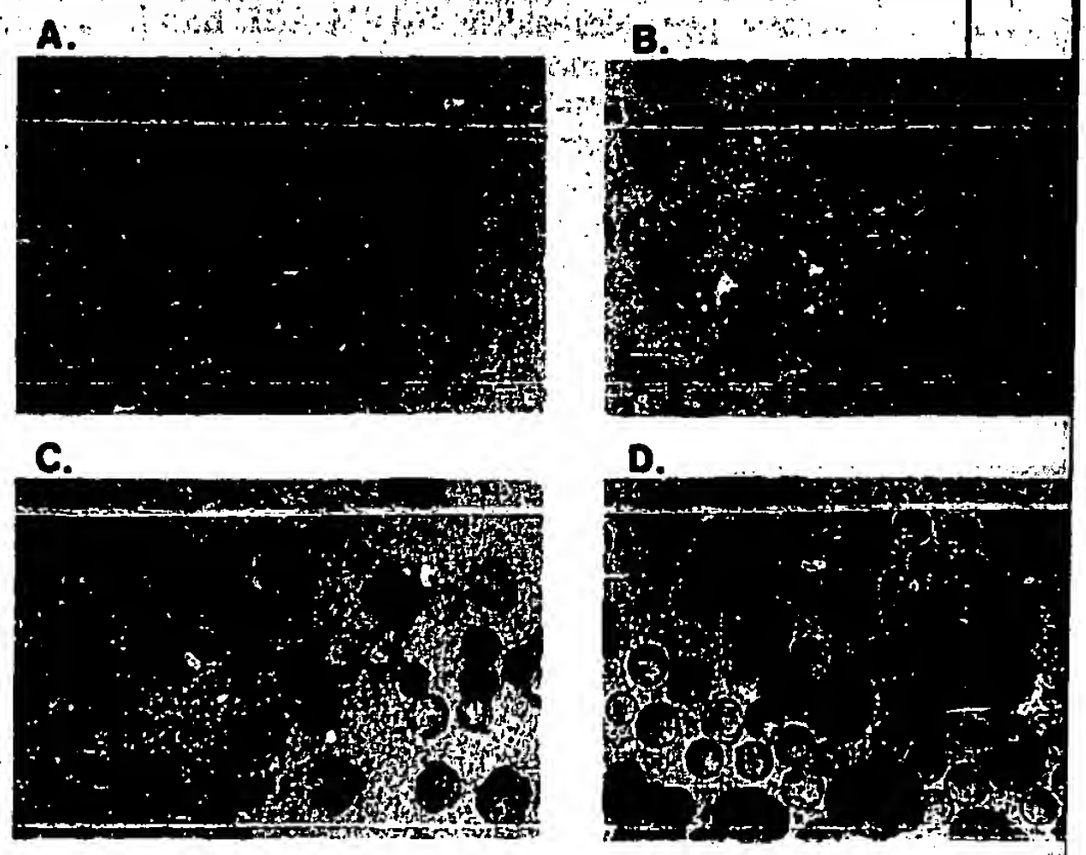

FIGURE 3 Morphology of CHO cells (40× magnification) grown in continuous perfusion culture. (A) day 3, (B) day 9, (C) day 18, (D) day 29.

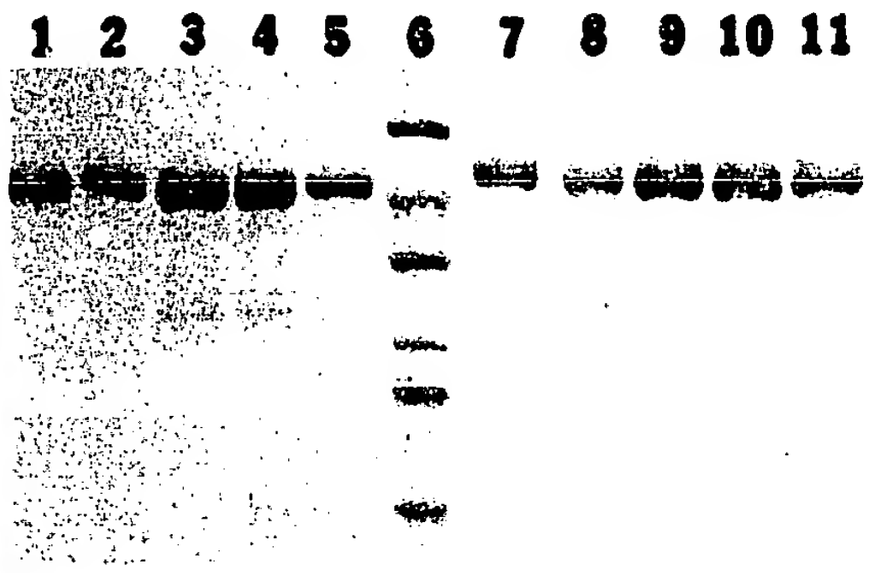

FIGURE 4 SDS-PAGE of CHO cell derived rscu-PA purified from conditioned medium harvested from the 6th week of fermentation. rScu-PA was purified by four column process consisting of S-sepharose (lanes 2, 8), ProZorb (lanes 3, 9), Mono-S (lanes 4, 10), ProZorb Ultra (lanes 5, 11). Scu-PA purified as above from TCL-598 cells (lanes 1, 7) MW markers (lane 6). Electrophoresis under reducing and non-reducing conditions is shown in lanes 1–6 and lanes 7–11 respectively.

weight on SDS-PAGE of 52 kD, and migrated with the same mobility as natural scu-PA isolated from the human kidney cell line TCL-598 (Fig. 4). The specific amidolytic activity of the rscu-PA isolated from CHO cells was 687 IU/mg. The specific amidolytic activity increased to greater than 140,000 IU/mg after pretreatment with plasmin. Amino acid sequencing of the purified material revealed an N-terminal amino acid sequence of ser-asn-glu-leu-his-gln-, which is consistent with the known amino acid sequence of urinary urokinase[27,28]. rScu-PA isolated from seven successive weekly harvests of conditioned medium obtained from a typical fermentation was shown to be similar with respect to purity and activity (data not shown).

The *in vitro* clot lysis activity of the rscu-PA was compared with that of scu-PA from TCL-598 cells and found to be identical (data not shown)[29]. *In vivo* thrombolytic activity in both a rabbit jugular venous thrombosis model and a dog coronary arterial thrombosis model, was also determined. The results demonstrated no significant difference between the natural and recombinant scu-PA molecules[30,31].

EXPERIMENTAL PROTOCOL

Cloning and expression of the human cDNA gene for scu-PA. The scu-PA cDNA gene was cloned from human TCL-598 kidney cells as we have described previously[27]. The transfection and amplification of scu-PA in CHO cell line DG-44 was also accomplished as described elsewhere [6,32].

Spin filter. A 7.6 cm diameter by 25 cm long 127 mesh ETFE rotating spin filter (FlourTex monofilament), was obtained from Hub Wire Cloth in Everett, MA. The screen was attached to a support structure with silicone adhesive (General Electric GE#5000). The support structure consisted of 4 vertically oriented 0.1×0.5 cm stainless steel bars welded to two horizontally oriented circular hubs designed to fit over the driveshaft. The completed assembly, when fitted to the driveshaft (Fig. 1), formed a rotating "cup" with watertight stainless steel bottom, screen sides and open top. The sieve was positioned on the drive shaft so as to have 5 cm of screen extending above the normal liquid operating level of the fermentor. A 4.8 inch diameter axial-flow impeller (Mixing Equipment Co.) was positioned 5 cm below the sieve on the driveshaft and the whole assembly was operated at 150 RPM.

Cell growth. Cells were routinely propagated in T-flasks and roller bottles in DMEM:F12(1:1) medium with 2% FBS and 20 μM methotrexate. Fermentors were inoculated with cells harvested from roller bottles by trypsinization and resuspended in a 3 liter spinner flask (Bellco) containing 2,700 ml of serum-free CRI-G3 medium with 50 grams of microcarrier beads (Cytodex-2, Pharmacia). After a 2–3 hour incubation at 37°C and 20 RPM, the mixture was transferred to a fermentor containing 7 liters of medium supplemented with 0.5% FBS. Fermentations were perfused as indicated with CRI-G3 medium. CRI-G3 medium contains DMEM:F12 (1:1, Gibco, Grand Island, N.Y.) supplemented with 0.5% FBS and 5 mg/l insulin (#40305-Collaborative Research, Bedford, MA), 5 mg/l bovine transferrin (#82-056 Miles, Kankakee, IL), 200 mg/l linoleic acid/bovine serum albumin (BSA) (#40227-Collaborative Research, Bedford, MA), 100 mg/l Pluronic F-68 (BASF, Parsippany, N.J.), 0.5 ml/l Ex-cyte (Miles, Kanakee, IL) and 5× MEM vitamins (Gibco, Grand Island, NY). Medium used for perfusion with clone CGM-113 was supplemented with 5 μM methotrexate (Sigma, St. Louis, MO.). Viable cell counts were determined from samples resuspended in 0.05% trypsin-0.53 mM EDTA at room temperature for 30 minutes and diluted to 20% FBS. Trypan Blue (Gibco, Grand Island, NY) was added and single cells were counted by hemocytometer. The extent of cell lysis in the fermentor was estimated by measuring the concentration of lactate dehydrogenase and phosphohexose isomerase (Sigma procedures 228-UV and 355-UV) in cell free conditioned medium (centrifuged at 1,200×G, 10 min.) and in sonicated cell supernatants. Sigma enzyme control E-5 was used for an internal standard[33,34]. The specific cell lysis rates were calculated by: dilution rate×extracellular enzyme level/liter reactor volume/intracellular enzyme level/liter of reactor volume. The size of cells and cell clumps was estimated by direct microscopic observation against a calibrated hemocytometer grid.

Purification of rscu-PA. Conditioned medium from fermentations was filtered and purified over S-Sepharaose Fast Flow (Pharmacia, Piscataway, N.J.) and *p*-aminobenzamidine (pABZ) sepharose (ProZorb, Collaborative Research, Bedford, MA) as described elsewhere[32]. The pABZ Sepharose eluate was applied to a Mono-S column (Pharmacia, Piscataway, NJ), equilibrated with 0.02 M sodium acetate (pH 5.3), 0.1 M sodium chloride and rscu-PA was eluted from the column with a linear gradient to 1 M sodium chloride. The main peaks of protein were pooled, diluted three-fold with 0.02 M sodium acetate and applied to a second pABZ-Sepharaose (ProZorb Ultra, Collaborative Research, Bedford, MA) column, equilibrated with 0.092 M sodium acetate (pH 5.3), 0.2 M sodium chloride, to remove traces of tcu-PA. Scu-PA does not bind to ProZorb Ultra under these conditions while tcu-PA does. Therefore, the main peak of protein passing through the column was pooled, concentrated by ultrafiltration, and stored frozen at −20°C. The activity of scu-PA and tcu-PA in conditioned medium and column fractions was measured by determining the amidolytic activity on the colorimetric substrate S-2444 (Kabi-Vitrum, Stockholm, Sweden) before and after treatment with plasmin as has been described elsewhere[32]. A specific activity of 140,000 international units per mg of protein was used to convert to protein concentrations. SDS polyacrylamide gel electrophoresis was performed with 12% polyacrylamide gels using the buffer system of Laemmli[35]. Protein was quantitated by the method of Lowry et al[36].

Acknowledgments

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